Control of *Plum pox virus* through the use of genetically modified plants

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Genetic resistance to *Plum pox virus* (PPV) is the most viable alternative for long-term control of sharka disease. In addition to the classical approaches to producing resistant germplasm and cultivars, genetic transformation offers a promising genetic approach to resistance. We show, using the example of C5 plum, that genetically engineered resistance can provide durable, stable and high levels of PPV resistance. A review of the results of work with C5 including molecular analyses of resistance and long-term field testing is presented.

Introduction

Plum pox virus (PPV, genus *Potyvirus*), causing sharka disease of *Prunus*, is responsible for extensive economic losses (Németh, 1994; Roy & Smith, 1994). Control of PPV has been chiefly through quarantine and eradication of infected trees. These measures have been insufficient to control the spread of PPV within and between countries and continents. The use of resistant cultivars is a critical control strategy that remains to be fully implemented. There are two approaches to the development of resistant cultivars. Natural PPV resistance may be exploited by the identification of resistant genotypes followed by the transfer of resistance genes into new germplasm through hybridization. To assure the durability of resistance and high levels of fruit quality necessary for the market, promising resistant selections must then be extensively field-tested before release to growers. Genomic studies and genetic markers can speed the selection of putative resistant seedlings and this approach is currently being pursued (Abernathy *et al*., 2004). Even then, the process from hybridization to cultivar release can span decades. Alternatively, resistant cultivars can be obtained by use of transformation with genes for PPV resistance.

Transgenic plants for virus resistance

The engineering of viral genome segments and their use as resistance genes has been demonstrated (Baulcombe, 1996). This process, termed pathogen-derived resistance (Sanford & Johnston, 1985), may be used to improve existing economically important cultivars assuming that these cultivars are amenable to transformation. Transformation of existing cultivars holds promise for the relatively rapid deployment of resistance. Alternately, if resistance genes are transformed into seedling plants, as in the case of plum (Scorza *et al*., 1994), these genes can be used in the long term to produce new PPV resistant cultivars through the processes of hybridization and selection. Transgenes and naturally occurring resistance genes may also be combined through hybridization for broad-based multigenic resistance.

Transgenic plants expressing viral genes have been shown to exhibit varying degrees of resistance to viruses homologous or closely related to the source of the transgene (Beachy *et al*., 1990). Studies of viral transgene-mediated resistance have shown that resistance may either be mediated through the production of transgene protein (Wilson, 1993) or RNA-mediated. RNA-mediated resistance may take the form of post-transcriptional gene silencing (PTGS), also known as RNA silencing. PTGS has been associated with multiple transgene copies, particularly direct repeats of the transgene coding region (Dehio & Schell, 1994; Sijen *et al*., 1996), truncated or antisense copies of the transgene insert (Hamilton *et al*., 1998; Waterhouse *et al*., 1998), and methylation of the coding region (Ingelbrecht *et al*., 1994; Smith *et al*., 1994; English *et al*., 1996; Van den Boogaart *et al*., 1998; Guo *et al*., 1999; Jones *et al*., 1999; Sonoda *et al*., 1999). PTGS appears to be mediated at least in part by small 21–25 nucleotide RNAs (Hamilton & Baulcombe, 1999; Waterhouse *et al*., 2001; Llave *et al*., 2002). Non-PTGS plants containing sequences homologous to the PTGS sequence can become silenced when grafted onto a PTGS stock (Palauqui *et al*., 1997; Mlotshwa *et al*., 2002), an interesting finding considering that virtually all fruit varieties are grafted onto rootstocks. The use of 'silencing' rootstocks may be a feasible approach for the improvement of scion varieties without the need for transformation of these varieties. The above observations have formed the basis for several PTGS models that have been extensively reviewed and discussed (Smith *et al*., 1994; Baulcombe, 1996; Sijen *et al*., 1996; Waterhouse *et al*., 1998; Van den Boogaart *et al*., 1998; Hamilton & Baulcombe, 1999; Waterhouse *et al*., 2001).

Prunus transformation

In the early 1990s, the successful transformation of *Prunus* (Mante *et al*., 1991) led us to approach the control of PPV

through transgene-based resistance. A modified PPV coat protein (CP) gene construct designed to express CP was developed (Ravelonandro *et al*., 1992). The results of challenging transformed herbaceous plants demonstrated both a recovery reaction and the apparent immunity of transgenic plants (da Câmara Machado *et al*., 1992; Ravelonandro *et al*., 1993, 1994; Jacquet *et al*., 1998). These reactions were associated with a down-regulation of the transgene products (Ravelonandro *et al*., 1994; Jacquet *et al*., 1998) suggesting RNA-mediated sense suppression as the basis for resistance.

Following the successful development of PPV-CP transgenic PPV-resistant herbaceous plants, PPV-CP gene constructs were transferred into *Prunus* species (Laimer da Câmara Machado *et al*., 1992; Scorza *et al*., 1994). Transgenic plums (*Prunus domestica*) containing the PPV-CP transgene insert demonstrated various levels of resistance with the highest level shown by clone C5 (today named 'HoneySweet') which contained a multicopy insert and produced low levels of PPV-CP mRNA and no detectable PPV-CP (Scorza *et al*., 1994; Ravelonandro *et al*., 1997). Further molecular analyses of C5 demonstrated that this clone contained aberrant copies of the transgene insert, and that the PPV-CP gene was methylated and silenced. PTGS was indicated as the mechanism of resistance (Scorza *et al*., 2001a,b).

Inoculation studies of C5 have shown that this clone is highly resistant to the major serotypes of PPV including D, M, El Amar, and Sour Cherry (Ravelonandro *et al*., 2001b). The highly conserved C-terminus of the core region of the CP gene appears to be involved in the homology-dependent resistance of C5 (Ravelonandro *et al*., 2002a). Homology to this conserved region appears to be the basis for the broad range of resistance to PPV serotypes by C5.

Evaluation of the PPV resistance of C5

To evaluate the stability and durability of the PTGS-based PPV resistance of C5, plants were transferred to the field to be tested against infection by bud-grafting with infected buds and by aphid vectors under natural orchard conditions. These field tests were initiated under the appropriate permits in Bistrita (RO), Skierniewice (PL) and Valencia (ES) in 1995, 1996, and 1997, respectively. To date, following 10, 9, and 8 years, respectively, these field tests have shown that no C5 plants have been infected by natural aphid vectors when evaluated by ELISA, and RT-PCR, including immuno-capture RT-PCR. Trees inoculated by infected chip buds or rootstocks have shown only a very low level of infection generally only detected by RT-PCR and with transient symptoms on only a few leaves on a tree. These graft-inoculated trees appear to recover and it is difficult to either find symptoms or positive molecular indications of infection (Ravelonandro *et al*., 2002b; Hily *et al*., 2004; Malinowski *et al*., 2006).

Taking into account the frequent association of PPV with other fruit tree viruses (*Prunus necrotic ringspot* (PNRSV) and *Prune dwarf ilarviruses*, *Apple chlorotic leafspot trichovirus* (ACLSV) in the development of disease, we have begun to test the reaction of the PPV-resistant C5 plum to coinfection with other *Prunus* viruses. Results to date indicate that PPV can be detected at low levels in some C5 plants graft-inoculated with both PPV and PNRSV, or with both PPV and ACLSV (Polák *et al*., 2005; Ravelonandro *et al*., 2006). It is not yet clear whether PPV infection in C5 following mixed virus graft inoculations is higher than that produced by graft inoculation of PPV alone. Molecular studies are under way.

The detection of siRNA homologous to PPV sequences in C5 (Hily *et al*., 2005) not only further confirms the PTGS mechanism of resistance in this clone but also opens the possibility of the movement of a silencing signal (Mlotshwa *et al*., 2002) from C5 that may affect susceptible material grafted onto C5. Research in this area is under way. It is interesting to note that, upon inoculation with PPV, siRNA production was detected in nontransformed PPV-susceptible plums (Hily *et al*., 2005). The siRNA produced in these plants was of the 22-nt species which is thought to be involved in a localized silencing (Hamilton *et al*., 2002) while the resistant C5 produced both the 22 and a 25-26 nt species of siRNA, the longer species being associated with systemic silencing (Hamilton *et al*., 2002). This suggests that the production of siRNA in plum is a native form of resistance (Voinnet, 2001). C5 has been modified through genetic engineering to produce the longer species of siRNA, thereby improving the effectiveness of this native virus resistance mechanism.

Production of new PPV-resistant lines

While the development of a PTGS plum clone highly resistant to PPV demonstrates the utility of this technology for developing PPV resistance, it was important to evaluate the possibility of transferring resistance to new lines through hybridization. Hybrids between C5 and transgenic and nontransformed *P. domestica* clones (Ravelonandro *et al*., 1998, 2002c; Scorza *et al*., 1998), and between C5 and *P. spinosa* (Ravelonandro *et al*., 2001a) were produced. The multicopy insert in C5 acted as a single locus and this insert and the associated gene silencing and resistance to PPV were transferred to plum seedlings as a dominant gene trait. These studies demonstrated the utility of C5, a PTGS resistant transgenic clone, as a source of PPV resistance in plum breeding programmes. The relative ease of selecting resistant transgenic seedlings through GUS assays or PCR is an advantage in utilizing this clone to transfer resistance since these markers can be used to select resistant seedlings and thus reduce the time and space required for the breeding programme.

Future prospects and conclusions

Now that the potential utility of PTGS for PPV resistance in *Prunus* is clear, there are a number of areas of research that require attention in order to use this technology efficiently to produce PPV-resistant stone fruits that can be used by growers in areas infested by PPV. Several laboratories, including our own, are producing new gene silencing constructs, specifically

'hairpin' constructs (Smith *et al*., 2000; Wesley *et al*., 2001), utilizing CP and other PPV sequences. These transgenic plants will require rigorous glasshouse testing and field trials to verify the level, stability and breadth of resistance in terms of resistance to a broad range of PPV serotypes. Other research areas that are receiving attention include: 1) the development of efficient transformation systems for stonefruit species, especially peach, including clonal transformation systems for transformation of cultivars to provide for rapid improvement of PPV-susceptible cultivars that are currently grown in infected areas; 2) the development of gene constructs and promoters that may provide greater consumer acceptance of GMOs and aid in regulatory approval and grower utilization. Collaboration between virologists, breeders, transformation specialists and molecular biologists, nationally and internationally, will be important for the progress of all these research efforts.

Our results demonstrate that resistance to PPV through PTGS is highly efficient and broadly effective against many strains of the virus. Resistance has remained durable and stable in the field for 10 years. This technique is a promising approach not only for developing virus-resistant genotypes but also for the improvement of any number of important traits such as resistance to fungal and bacterial pathogens, insect resistance and the improvement of fruit quality and other horticultural traits.

Lutter contre le *Plum pox virus* en utilisant les plantes génétiquement modifiées

La résistance au *Plum pox virus* (PPV) est une approche alternative fiable pour lutter à long terme contre ce virus. Parallèlement à la génétique classique qui permet d'obtenir un cultivar résistant, la transgénèse offre une approche alternative prometteuse pour parvenir à cette fin. Nous démontrons, en utilisant l'exemple du prunier C5, que les manipulations génétiques permettent de conférer à la plante un haut niveau de résistance au PPV qui est à la fois durable et stable. Les résultats des travaux avec C5 sont développés ici, y compris les analyses moléculaires de résistance et les tests au champ.

Борьба с *вирусом шарки сливы* с помощью генетически модифицированных растений

Генетическая резистентность к *вирусу шарки сливы* (PPV) – самая надежная альтернатива для долгосрочной борьбы с этой болезнью. В дополнение к классическим подходам, таким как создание резистентных гермоплазмы и сортов растений, генетическое преобразование представляет собой перспективный подход к выработке устойчивости. На примере сливы C5 показано, что генетически созданная резистентность может обеспечить длительные, стабильные и высокие уровни резистентности в отношении PPV. В статье представлен обзор результатов работы с C5, включая молекулярные анализы резистентности и долгосрочные полевые испытания.

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